THE RELATIONSHIP BETWEEN MEMBRANE POTENTIAL AND Ca^{2+} FLUXES IN ISOLATED SARCOPLASMIC RETICULUM VESICLES

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1. Introduction

The absorption and fluorescence of cyanine, oxonol and merocyanine dyes are sensitive to changes in membrane potential [1]. Although the optical response is usually complicated by contributions from cation binding [2,3], under carefully defined conditions the probes are reliable indicators of membrane potential in subcellular particles and in living cells.

This report deals with the relationship between ion diffusion potential and the flux of calcium ions in sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle. The cyanine dye (Di-O-C_2(5)) was used as potential probe.

Two questions were investigated.

1. Do potential changes accompany the ATP-mediated active uptake [4] and the (ADP + P_i)-induced release of Ca^{2+} [5] by sarcoplasmic reticulum vesicles?
2. What is the influence of artificially-imposed membrane potential upon the rate of Ca^{2+} release from sarcoplasmic reticulum?

2. Experimental

2.1. *Materials*

Several oxocarbocyanine dyes were kindly provided by Dr Alan Waggoner of the Department of Chemistry, Amherst College, Amherst, MA. Methanesulfonic acid and Di-O-C_2(5) were obtained from Eastman Kodak, Rochester, NY. Egg phosphatidylcholine was purchased from Sylvana Chemical Co., Millburn, NJ, chlortetracycline from Sigma Chemical Co., St Louis, MO, and arsenazo III from Aldrich Chemical Co., Milwaukee, WI.

Abbreviations: KMS, K-methane-sulfonate; NaMS, Na-methane-sulfonate; TMS, Tris-methane-sulfonate; val, valinomycin; Di-O-C_2(5), 3,3'-diethyloxodicarbocyanine

2.2. *Procedures*

Microsomes were prepared from rabbit skeletal muscle as in [6]. For absorbance measurements an Aminco DW-2 spectrophotometer and for fluorescence studies the Aminco-Bowman spectrofluorometer or the Aminco DW-2 spectrophotometer equipped with fluorescence accessory were used. The uptake and release of Ca^{2+} by sarcoplasmic reticulum vesicles were measured by one or more of the following techniques: chlortetracycline fluorescence [7] Millipore filtration using ^{45}Ca [8], and arsenazo III titration [9]. Protein concentration was determined by the Lowry method [10] and phospholipid-P as in [11]. Liposomes were prepared as in [12].

3. Results

3.1. *Absorbance changes of Di-O-C_2(5) during Ca^{2+} transport and Ca^{2+} release by sarcoplasmic reticulum*

Addition of calcium at mM levels to a suspension of sarcoplasmic reticulum vesicles in the presence of Di-O-C_2(5) decreases the absorbance with a minimum at 600 nm (fig.1). A similar change in absorbance occurs during ATP-mediated Ca^{2+} uptake by microsomes at free medium $Ca^{2+} < 0.1$ mM. Calcium at this

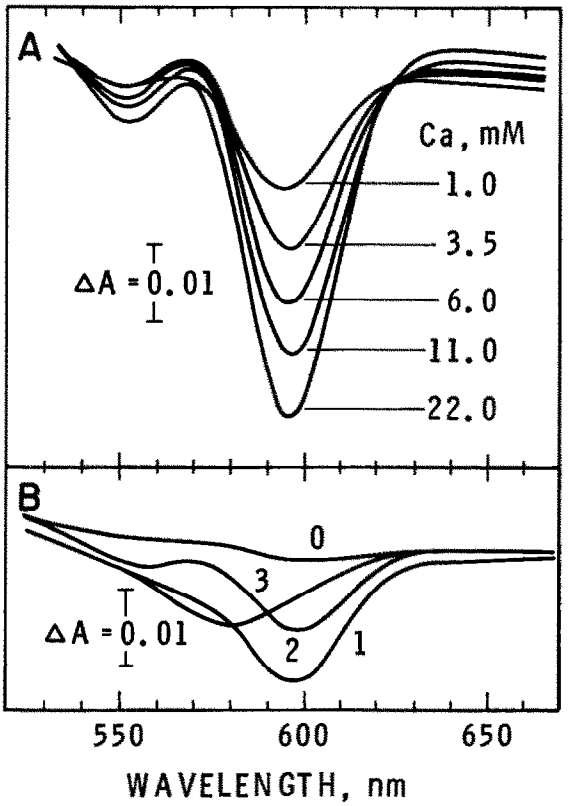

Fig.1. Effect of Ca^{2+} and ATP on the absorption spectrum of Di-O-C_2(5) in the presence of microsomes. (A) The medium contained 0.12 M KCl, 10 mM Tris–maleate (pH 7.0), 0.1 mM EGTA, 0.66 μg/ml Di-O-C_2(5), 66 μg microsomal protein/ml and in the sample cell, Ca^{2+} at final concentrations indicated in the figure. (B) The medium contained 0.12 M KCl, 10 mM Tris–maleate (pH 7.0), 4.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mg microsomal protein/ml, and 3 μg Di-O-C_2(5)/ml (line 0). To the sample cell, 3.3 mM ATP was added and the spectrum recorded about 1 min later (1). Then ADP (0.3 mM), P_i (4.5 mM) and EGTA (1.6 mM) were added to both reference and sample cells and the spectrum recorded 1 min (2) and 7 min (3) later.

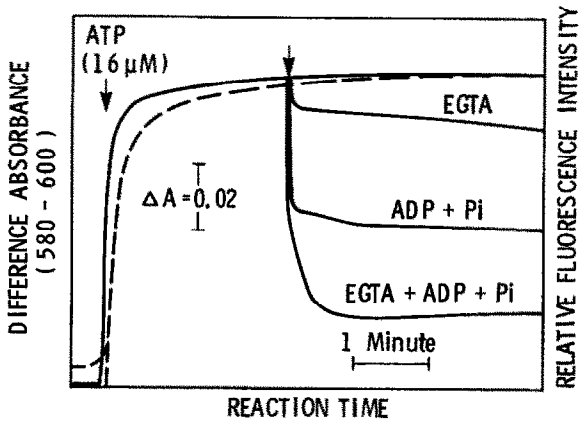

Fig.2. ΔA of Di-O-C_2 (5) during Ca^{2+} uptake and release by sarcoplasmic reticulum. Conditions are similar to those in fig.1B. At the first arrow ATP was added to a 16 μM final conc. At the second arrow EGTA (8.3 mM), inorganic phosphate (12.5 mM) and ADP (1.6 mM) were added in various combinations. Broken line indicates the rate of Ca^{2+} uptake measured by chlortetracycline fluorescence.

concentration produces only a slight effect in the absence of ATP. The time course of the ATP-induced absorbance change correlates with the rate of Ca^{2+} uptake (fig.2). Release of Ca^{2+} from sarcoplasmic reticulum upon addition of EGTA, ADP and orthophosphate is followed by reversal of the Di-O-C_2(5) response (fig.2). The absorbance change of Di-O-C_2(5) was also observed upon addition of Ca^{2+} at mM levels to sarcoplasmic reticulum preparations solubilized with deoxycholate. Under these conditions contribution of membrane potential is negligible. Therefore, in analogy with earlier observations on related cyanine dyes (2,3), the Di-O-C_2(5) response during Ca^{2+} uptake is attributed to binding of Ca^{2+} to membrane-linked cation binding sites. There is no indication of changes in membrane diffusion potential during Ca^{2+} uptake or release.

3.2. *The response of Di-O-C_2(5) to membrane potential generated by valinomycin*

A large change in the absorbance of Di-O-C_2(5) is observed in liposomes and in skeletal muscle microsomes in response to membrane potential generated by valinomycin (fig.3). The valinomycin-induced difference absorbance minimum is at 575 nm, in contrast with the 600 nm minimum obtained in the presence of Ca^{2+} or during ATP-induced Ca^{2+} transport. On the basis of this difference it is unlikely that the optical response of Di-O-C_2(5) during Ca^{2+} transport contains significant contribution by membrane potential. The magnitude of the valinomycin-induced optical response correlates reasonably well with the calculated membrane potential both on liposomes and on microsomes, above 50 mV (not shown). On microsomes the optical change is rapidly reversed due

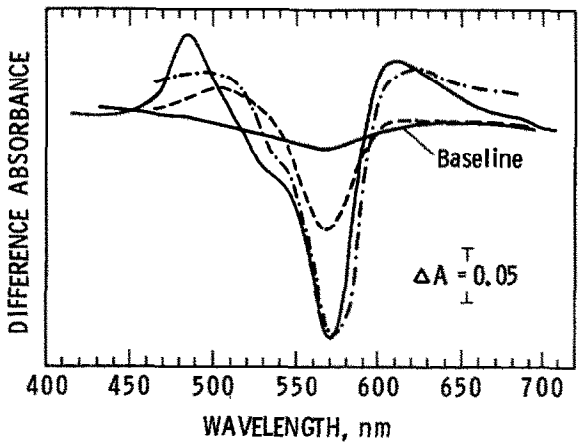

Fig.3. Difference absorbance of Di-O-C_2(5) in the presence of valinomycin. Vesicles containing 0.3 M KMS were diluted 300-fold into a medium of 0.3 M NaMS, 10 mM Tris–maleate (pH 7.0) and 1 μg/ml Di-O-C_2(5). To the sample cell, valinomycin was added to 0.58 μM final conc. and the difference absorbance measured against a valinomycin-free control. (———) Liposomes prepared from egg lecithin: cholesterol 3:1 (w/w), 25°C; (- - - -) sarcoplasmic reticulum vesicles (0.13 mg/ml), 4°C; (- · - · -) microsomal phospholipids, 20°C.

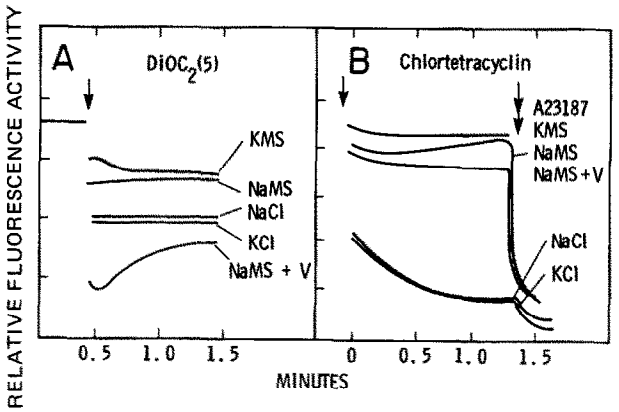

Fig.4. Membrane potential and the rate of Ca^{2+} release from sarcoplasmic reticulum vesicles passively loaded with Ca^{2+}. Microsomes (40 mg protein/ml) were equilibrated in 0.3 M KMS, 10 mM Tris–maleate (pH 7.0), 15 mM Ca-gluconate and 0.1 mM chlortetracycline overnight at 2°C. Aliquots (10 μl) were diluted (arrow) into 3 ml medium which contained 10 mM Tris–maleate (pH 7.0), 1 μg/ml Di-O-C_2(5) and 1 mM EGTA together with 0.3 M KMS, NaMS, KCl or NaCl, with or without 1 μM valinomycin (V). The changes in Di-O-C_2(5) fluorescence were recorded at 9°C using light of 550 nm and 600 nm for excitation and emission, respectively (left panel). Ca^{2+} release measurements with chlortetracycline as indicators were carried out at excitation and emission wavelengths of 390 nm and 530 nm, respectively, under the same conditions (right panel). At the double arrow A23187 (1 μM) was added to release remaining Ca^{2+}.

to the leakage of anions and cations through the membrane. The potential signal was stable for several minutes on liposomes.

3.3. *The relationship between membrane potential and the rate of Ca^{2+} release from sarcoplasmic reticulum*

Dilution of Ca^{2+}-loaded sarcoplasmic reticulum vesicles equilibrated with K-methanesulfonate into isoosmotic KCl solution causes the rapid release of calcium [13–15]. The Ca^{2+} release is usually attributed, without experimental evidence, to an inside negative membrane potential generated by the fast entry of Cl^- into the vesicles [16], although the effect of osmotic swelling was also considered [13–15]. The purpose of these studies is to determine the relationship between the rate of Ca^{2+} release and the magnitude and direction of artificially-imposed membrane potential on sarcoplasmic reticulum vesicles.

Muscle microsomes were equilibrated with 0.3 M K-methanesulfonate and 15 mM Ca-gluconate and diluted into media containing methanesulfonate or chloride salts of Na^+, K^+ or other monovalent cations. Large change in potential was generated upon dilution into media containing Na-methanesulfonate and valinomycin (fig.4A), with only slight change in the rate of Ca^{2+} release (fig.4B). Dilution into 0.3 M KCl or 0.3 M NaCl in the absence of valinomycin caused rapid release of Ca^{2+} as monitored by chlortetracycline (fig.4B), with only small potential change (fig.4A). Essentially identical results were obtained using ^{45}Ca and Millipore filtration for measurement of the Ca^{2+} fluxes.

The contribution of Ca^{2+} diffusion potential to the observed response is assumed to be negligible since similar potential changes were observed on Ca^{2+}-free and on Ca^{2+}-loaded vesicles.

The correlation between inside positive membrane potential and the rate of Ca^{2+} release from sarcoplasmic reticulum was also tested by equilibrating microsomes in 0.3 M Na-methanesulfonate and diluting them into media of 0.3 M K-methanesulfonate containing 1 mM EGTA, with or without 1 μM valinomycin. The rates

of Ca^{2+} release were similar in dilution media containing 0.3 M K-methanesulfonate or 0.3 M Na-methanesulfonate with or without valinomycin (not shown).

Therefore, the increased rate of Ca^{2+} release upon dilution of vesicles from methanesulfonate into chloride media is not related to changes in membrane potential. The chloride-induced Ca^{2+} release is inhibited in hypertonic media containing 0.2–0.6 M sucrose or 0.1–0.3 M K-methanesulfonate, in addition to 0.3 M KCl. These observations confirm earlier suggestions [13–15] that osmotic swelling of the vesicles contributes to the chloride-induced Ca^{2+} release. Direct influence of chloride upon the interaction of sarcoplasmic reticulum membranes with Ca^{2+} seems unlikely as choline chloride did not induce Ca^{2+} release (not shown) and the amount of Ca^{2+} bound to sarcoplasmic reticulum vesicles after passive equilibration with 0.1–5.0 mM $^{45}CaCl_2$ was similar in K-methanesulfonate and KCl media.

4. Discussion

The optical changes of Di-O-C_2(5) during the ATP-mediated uptake or the (ADP + P_i)-induced release of calcium by sarcoplasmic reticulum vesicles provided no indication that either the Ca^{2+} uptake or the Ca^{2+} release is electrogenic. Electroneutrality may be maintained during active Ca^{2+} transport by anion fluxes [6] or through counter transport of Mg^{2+} [17] and other cations.

Dilution of Ca^{2+}-loaded sarcoplasmic reticulum vesicles from K-methanesulfonate into KCl medium increases the rate of Ca^{2+} release [13–15]. Based on earlier studies on skinned muscle fibers it was assumed that the Ca^{2+} release follows a wave of inside negative potential generated by rapid influx of Cl^- into the vesicles [16]. Our measurements with Di-O-C_2(5) do not support this interpretation. As generation of large inside negative or inside positive potential by valinomycin in a methanesulfonate medium had no influence upon the rate of Ca^{2+} release, it is unlikely that the much smaller potential changes upon transfer of vesicles from K-methanesulfonate into KCl media could explain the very fast Ca^{2+} release observed under these conditions. These observations emphasize the importance of local changes at the triad in the activation process.

Further work is required to decide whether a change in potential across sarcoplasmic reticulum membranes occurs during contraction of living muscle and if it contributes to Ca^{2+} release.

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